ELSEVIER

Bone Vol. 21, No. 2
August 1997:171–182

Pathogenesis of Vitamin (A and D)-Induced Premature Growth-Plate Closure in Calves

J. C. WOODARD,[1] G. A. DONOVAN,[2] and L. W. FISHER[3]

[1] *Department of Pathobiology and* [2] *Large Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL, USA*
[3] *Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, MD, USA*

The pathogenesis of vitamin A-induced premature growth-plate closure was investigated in calves. A progressive increase in the severity of growth-plate lesions with time and a progressive increase in the extent of growth-plate involvement was observed. There was initial loss of metachromasia from the growth plate in a region that formed a narrow horizontal band of cartilage composed of the epiphyseal growth zone and a strip of reserve-zone cartilage. Immunostaining revealed there was loss of aggrecan, decorin, and biglycan from this region; however, it was doubtful that the regional loss of proteoglycan was a major contributing factor in the pathogenesis of premature growth-plate closure. This is because this region was the vestige of cartilage that remained when growth-plate closure was almost complete. The major alteration was premature mineralization of columnar cartilage and subsequent endochondral ossification. This caused the depth of the columnar zone to be reduced. Columnar-zone cartilage cells appeared immature where the matrix became mineralized and lacked the morphology of hypertrophic chondrocytes. The depth of the reserve-cartilage zone also was reduced as matrix mineralization of the columnar zone progressed, and further reduction in columnar cartilage depth occurred. Eventually, there was matrix mineralization within the adjacent reserve cartilage. The distribution of reaction product after immunostaining with antibodies to the following proteins was described during normal endochondral ossification: aggrecan, decorin, biglycan, versican, type I collagen propeptide, type I collagen, type II collagen, osteopontin, osteocalcin, osteonectin, bone sialoprotein, and alkaline phosphatase. Biglycan, type I collagen propeptide, type I collagen, osteopontin, osteocalcin, osteonectin, bone sialoprotein, and alkaline phosphatase were localized within the cytoplasm or surrounding matrix of hypertrophic chondrocytes. In vitamin-treated calves, these same proteins were found in regions undergoing premature matrix mineralization even though the chondrocytes did not have a hypertrophic morphology. Therefore, vitamin treatment did not cause just a selective expression, but it caused expression of a large number of matrix proteins normally associated with the hypertrophic chondrocyte phenotype. Finally, completely mineralized columnar and reserve cartilage were removed by a modeling/remodeling process similar to that seen in the metaphysis. (Bone 21:171–182; 1997)

Key Words: Vitamin A; Premature growth-plate closure; Bone and cartilage matrix proteins; Immunohistochemistry; Premature chondrocyte mineralization; Chondrocyte differentiation.

Introduction

Hyena disease is an uncommon spontaneous disease of young dairy cattle that causes dwarfism in which the caudal body structures are relatively underdeveloped. Conformational features that cause cattle to have a hyena-like appearance occur because there is premature closure of the growth plate. We reported cases of hyena disease at a Florida dairy farm where animals were given injections of vitamins A and D.[28] Experimental studies demonstrated that vitamins A and D injected into newborn calves, and subsequent oral administration of vitamin A, caused focal growth-plate closure that began within 1 week.[44] The microscopic changes within the growth plate demonstrated localized loss of cartilage metachromasia in a portion of the reserve cartilage. Longitudinal bone growth was inhibited in the affected bone, and there was premature endochondral ossification of the columnar cartilage. Various effects of vitamin A or retinoic acid have been demonstrated in tissue culture, which include degradation and loss of proteoglycan from cartilage[19] and alteration in chondrocyte gene expression for matrix proteins.[4,23,34] The purpose of the current study was to define the cellular and matrix alterations that occur during premature growth-plate closure in calves and to determine whether the observed cartilage alterations were similar to those induced by retinoic acid in vitro.

Materials and Methods

Animals

Day-old bull calves that had received colostrum and had a plasma protein greater than 5.5, as measured by a refractometer, were purchased and housed outdoors in single portable wire-pen enclosures.

Animals were fed a milk substitute twice daily and were given a calf supplement ad libitum. Animals were weighed at birth and weekly thereafter. Four control and six vitamin-treated animals made up the experimental group. One treated and one control animal were killed at the end of weeks 1 and 2, and two

Address for correspondence and reprints: Dr. J. Carroll Woodard, Department of Pathobiology, College of Veterinary Medicine, University of Florida, P.O. Box 100145, Gainesville, FL 32610-0145.

This study is journal series article No. 510, College of Veterinary Medicine, University of Florida.

8756-3282/97/$17.00
PII S8756-3282(97)00099-9

Bone Vol. 21, No. 2
August 1997:171–182

treated and one control animal were killed at the end of weeks 3 and 5.

Vitamin Treatments

The milk substitute provided the National Research Council's recommended daily allowance for dairy cattle of 13 IU vitamin A/kg per day. Treated animals were given an intramuscular injection of vitamins A and D on the first day after birth (4 mL of vitamin AD injectable, Vet Products Co., Lenexa, KS; 500,000 IU of vitamin A and 75,000 IU of vitamin D_3/per milliliter). In addition, treated calves were administered 50,000 IU of vitamin A per kilogram of body weight in a water dispersible form (ICN Pharmaceuticals, Inc., Cleveland, OH) added to the milk substitute daily. The amount of vitamin added to the milk substitute was adjusted weekly according to body weight.

Necropsy

At necropsy, the long bones were examined in all animals. The right proximal tibiae were sectioned longitudinally, perpendicular to the median plane, and a 0.3 cm central slab was obtained. The central portion of the growth plate was separated from most of the surrounding cancellous bone with a sharp blade.

Tissues

Bone tissues were fixed in 4% buffered paraformaldehyde (pH 7.2) for 24 h and placed in 70% ethanol. Growth-plate specimens from animals at each time period were decalcified in 10% neutral buffered ethylenediaminetetraacetic acid and embedded in paraffin. Other tissues were embedded undecalcified in methylmethacrylate containing dibutylphthalate. Tissue sections of undecalcified bone were cut using a Reichert-Jung Polycut S microtome (Leica, Deerfield, IL) and a tungsten-carbide D profile knife. All sections were placed on silanized slides, and incubated in a 55°C oven overnight.[20]

Histochemistry

Undecalcified, methylmethacrylate-embedded tissue sections were stained by von Kossa's method to localize mineral or toluidine blue to detect metachromasia.[1] Tissues for immunostaining were deparaffinized and rehydrated. All slides were enzymatically pretreated to enhance immunoreactivity. For this purpose, slides were incubated with chondroitinase ABC (from *Proteus vulgaris*, EC 4.2.2.4, Sigma, St. Louis, MO) for 15 min, followed by incubation with proteinase K (from *Tritirachium album*, EC 3.4.21.64, Sigma) for 30 min at 37°C before immunostaining. Sections were washed with phosphate-buffered saline (PBS) three times and incubated for 20 min in blocking buffer [150 μL normal goat serum per 10 mL of phosphate-buffered saline (PBS)]. The test sections were incubated with the following dilution of primary antibody in 1× buffer: aggrecan, 1:500; decorin, 1:500; biglycan, 1:1000; versican, 1:500, type I collagen, 1:1000; type I collagen propeptide, 1:1000; type II collagen, 1:1000; osteopontin, 1:500; osteocalcin, 1:1000; osteonectin, 1:2000; bone sialoprotein, 1:000; and alkaline phosphatase, 1:000. Controls consisted of substitution of the primary antibody with normal rabbit serum. All slides were incubated for 1 h at room temperature or 1 h at 37°C. Slides were washed and incubated with a 1:200 dilution of the appropriate biotinylated secondary antibody (biotinylated goat antirabbit IgG) for 30 min at room temperature. Antigen-antibody complexes were visualized when secondary antibody was coupled with avidin-biotin complexes using a commercial kit (Vector, Burlingame, CA). A diaminobenzidine-metal mixture was used as chromogen (Pierce, Rockford, IL).

Antibodies

Antibodies used in this study have been described elsewhere.[12,13,31,40] They include: aggrecan (Poole); bovine decorin, LF-94, -95; bovine biglycan, LF-96, -97; human versican, LF-99; bovine osteonectin, BON-I; bovine bone sialoprotein; bovine osteopontin, LF-124; bovine osteocalcin (Biomedical Technologies, Inc., Stoughtom, MA); bovine type I collagen (Chemicon International, Inc., Temecula, CA); bovine type II collagen (Chemicon); human procollagen I (Biogenesis, Sandown, NH); and bovine alkaline phosphatase, LF-52.[15]

Depth of Growth-Plate Cartilage Zones

The morphology of the proximal tibial growth plate of the calf can be divided into distinct zones. Two regions contribute to longitudinal bone growth in opposite directions. On the epiphyseal side, a small number of hypertrophic cartilage cells subjacent to the secondary ossification center contribute to longitudinal bone growth in a manner similar to hypertrophic cartilage cells on the metaphyseal side of the growth plate. This region is sometimes termed the articular-epiphyseal complex cartilage (A-E complex).[42] The growth region on the metaphyseal side is composed of cartilage cells arranged in rows and columns (zone of columnar cartilage). The columnar cartilage traditionally has been divided into a zone of proliferative cartilage, a zone of maturing and hypertrophic cartilage, a zone of calcified cartilage, and a zone of cartilage removal as the primary spongiosa is formed. The reserve cell zone is between the two growth zones.

The depth of various cartilage zones was measured optically on toluidine-blue-stained slides of growth plate using a digitizing analyzing system. The system consisted of a light microscope, a digitizing pad, and a microcomputer. The software program for measuring the depth of growth-plate cartilage zones (SCOPE©) was developed at the University of Florida (Computer Science Division, College of Medicine). In the 1 week control calf, the depth of the following cartilage zones were measured: total growth plate, epiphyseal growth zone (A-E complex), reserve cell zone, reserve cell zone plus the epiphyseal growth zone (total reserve zone), and the columnar cartilage zone. In the 1-week treated animal, measurements were made in three different locations: (1) where the growth plate had a nearly normal depth; (2) where the depth was moderately decreased; and (3) where the growth plate was extremely thin. The total depth of the growth plate was measured as the distance between the chondro-osseous junctions on the epiphyseal and metaphyseal sides of the growth plate. A region, termed the total reserve cartilage zone, was measured as the distance between the epiphyseal chondro-osseous junction and the margin of the reserve cartilage zone adjacent to where the cartilage cells began forming columns. This region included the depth of the small number of hypertrophic cartilage cells on the epiphyseal side of the reserve cartilage zone. The depth of columnar cartilage was the distance between the margin of the reserve cartilage zone and the metaphyseal chondro-osseous junction.

Results

By 5 weeks of treatment, all treated animals had growth plate lesions and showed gross evidence of premature growth-plate closure. There were microscopic lesions as early as 1 week. The light-microscopic findings in vitamin A-induced growth-plate

closure were presented previously.[11] In brief, prior to complete closure of the growth plate, there was loss of metachromasia in a portion of the reserve cartilage zone. The growth plate became extremely thin. There was premature endochondral ossification in which chondrocytes undergoing mineralization did not appear as hypertrophic chondrocytes. More details are given where applicable.

Because there was a progressive alteration in the severity of lesions and a progression in the extent of growth-plate involvement with time, findings are presented at weekly intervals. Because the localization of antibodies for matrix proteins used in this study have not been completely described for the bovine growth plate, a complete description is given for the week 1 control animal and, subsequently, differences between the treated and control animals are described.

Week 1: Control

Histology showed normal growth plate appearance, and von Kossa stains demonstrated a uniform front where hypertrophic chondrocytes were undergoing mineralization and where endochondral ossification was taking place (**Figure 1**A). The depths of various cartilage zones are shown in **Table 1**. The columnar cartilage zone was moderately thicker than the reserve cartilage zone (ratio 0.7 in the typical region; Table 1).

Immunohistochemistry. Staining of growth-plate tissues using normal rabbit serum in place of the primary antibody demonstrated only slight background staining. There was slightly increased staining of calcified cartilage matrix and the cartilage spicules of the primary spongiosa. Positive staining by antisera is reported only when the results were substantially stronger than the background levels.

Aggrecan. Heavy immunostaining of the chondrocyte cytoplasm, chondrocyte capsule, and territorial and interterritorial cartilage matrix was noted in all growth-plate zones (**Figure 2**A). There was heavy staining of calcified cartilage spicules in the primary spongiosa and in the cartilage cores of trabeculae. There was staining of cytoplasm of various bone and marrow cells.

Decorin. The most intense staining occurred in the reserve zone with dense staining of reserve-zone chondrocyte cytoplasm and moderate accumulation within the interterritorial matrix of this region (**Figure 3**A). Stain intensity of the matrix was decreased in the proliferative and maturing zone of the columnar cartilage, and cytoplasmic staining increased moderately within the hypertrophic cartilage zone. The chondrocyte capsule of hypertrophic cells was heavily stained. Osteoblasts and osteoclasts were stained. Bone surfaces were heavily stained, and stained fibrillar processes extended into the bone.

Biglycan. Chondrocyte cytoplasm of reserve zone chondrocytes stained intensely, but growth plate matrix was only minimally stained. The amount of matrix staining increased in the region of hypertrophic chondrocytes and in cartilage spicules at the chondro-osseous junction (**Figure 4**A). Osteoblasts were moderately stained, and osteoid surfaces beneath osteoblasts had a thin line of moderately stained matrix. Cytoplasm of marrow cells and endothelial cells were stained, and there was staining of vascular muscle cells in a perinuclear location.

Versican. Overall staining was light. Slight staining was evident in cytoplasm and territorial matrix of cells of the reserve cartilage. Localization was not evident in columnar cartilage cells, but territorial matrix staining was present. The staining intensity increased in the hypertrophic zone where there was

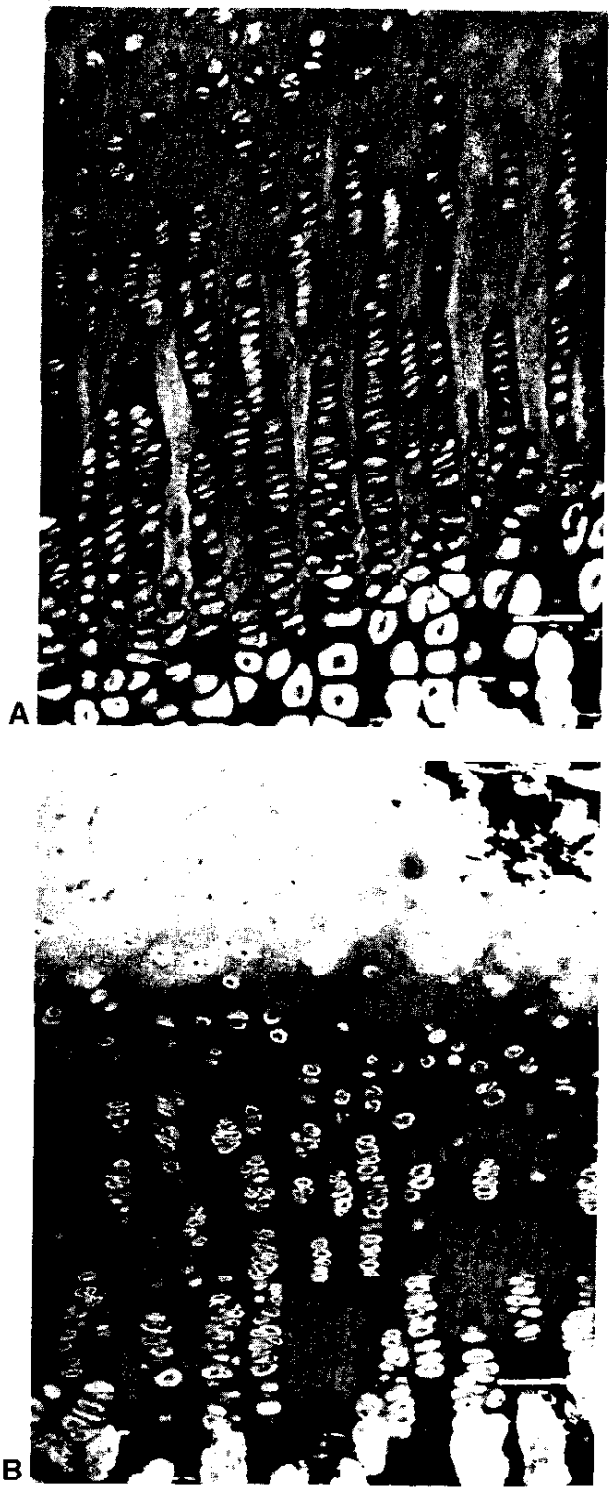

Figure 1. (A) Growth-plate/metaphyseal junction from a control calf. Note the size of the hypertrophic chondrocytes in the mineralized cartilage zone. Mineralized areas appear black. von Kossa tetrachrome stain; bar = 150 μm. (B) Growth-plate/metaphyseal junction from a vitamin A-treated calf. There are fewer columnar chondrocytes, and the terminal chondrocytes at the metaphyseal junction are smaller than normal. von Kossa tetrachrome stain; bar = 150 μm.

Bone Vol. 21, No. 2
August 1997:171–182

Figure 2. (A) Growth-plate subepiphyseal cartilage from a control calf stained to show distribution of aggrecan. Note dense pericellular matrix staining of subepiphyseal hypertrophic chondrocytes and matrix of the reserve cartilage zone. Bar = 80 μm. (B) Growth-plate subepiphyseal cartilage from vitamin A-treated calf stained to show distribution of aggrecan. Note loss of aggrecan localization around subepiphyseal hypertrophic chondrocytes and from the proximal matrix of the reserve cartilage zone. Bar = 80 μm.

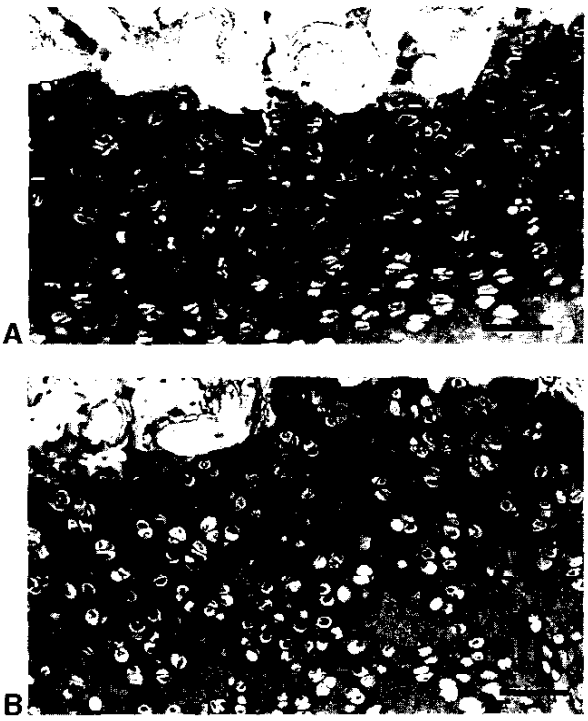

Figure 3. (A) Reserve cartilage zone from a control calf stained to show distribution of decorin. Staining is seen in chondrocyte cytoplasm and interterritorial matrix. Bar = 80 μm. (B) Reserve cartilage zone from a vitamin A-treated calf stained to show distribution of decorin. Stain intensity is diminished focally. Bar = 80 μm.

staining of interterritorial matrix (**Figure 5**A). Osteoid and bone were poorly stained.

Type I collagen. Osteoblasts were lightly stained. Osteoid covering cartilage spicules within the primary spongiosa were stained, and bone was uniformly stained. Chondroid cores within trabeculae and most of the growth-plate cartilage were unstained. There was slight staining around the hypertrophic chondrocytes (**Figure 6**A).

Type I collagen propeptide. Cytoplasm of the epiphyseal hypertrophic chondrocytes and cytoplasm of chondrocytes of the reserve cartilage zone stained heavily. Cells of the columnar cartilage cells were poorly stained, but increased staining was noted in chondrocyte cytoplasm as cells became hypertrophic. The interterritorial matrix of the columnar cartilage was moderately stained, and the stain intensity diminished and was limited to the chondrocyte capsule and cytoplasm in the hypertrophic cartilage zone (**Figure 7**A). Osteoblasts and newly formed os-

Table 1. Growth-plate depths (1 week calves)

		Treated[a]		
	Control	Wide	Medium	Narrow
Growth-plate depth (μm)	1029 ± 115	929 ± 172	649 ± 69	354 ± 71
A-E complex depth[b] (μm)	90 ± 20			
Reserve-zone depth (μm)	366 ± 103			
Total reserve zone depth[c] (μm)	456 ± 99	623 ± 164	401 ± 80	183 ± 55
Columnar zone depth (μm)	573 ± 65	304 ± 46	248 ± 48	170 ± 53
Ratio[d]	0.8	2.1	1.7	1.1

[a]Measurements made along lesion sites where growth plate varied in thickness.
[b]Depth of hypertrophic cartilage cells on the epiphyseal side of the growth plate. This region could not be clearly distinguished in the vitamin A-treated calf.
[c]Reserve zone depth plus the A-E complex zone depth.
[d]Ratio of depth of total reserve zone to columnar cartilage zone.

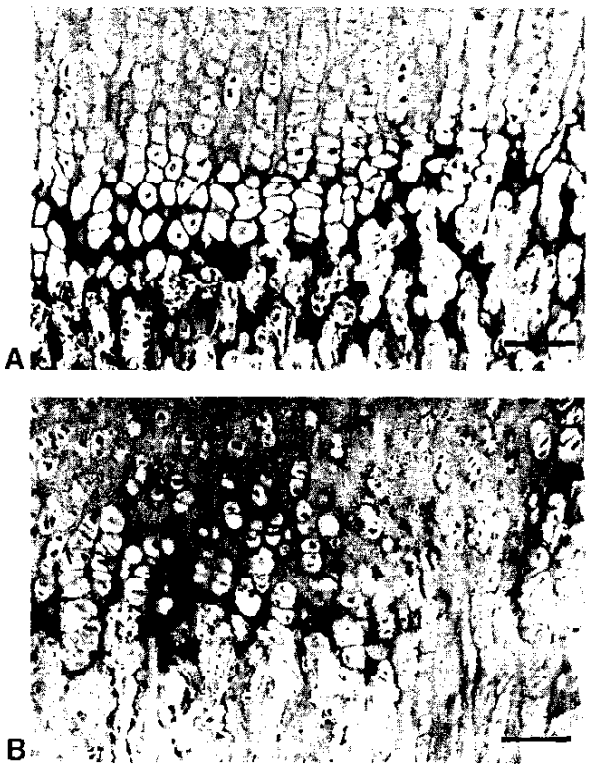

Figure 4. (A) Growth-plate/metaphyseal junction from a control calf stained to show the distribution of biglycan. Reaction product is present within chondrocytes and matrix of the hypertrophic chondrocyte zone and within osteoblasts and on osteoid surfaces lining trabeculae of the primary spongiosa. Bar = 80 μm. (B) Growth-plate/metaphyseal junction from a vitamin A-treated calf shows the distribution of biglycan to be similar to that of the control animal (A).

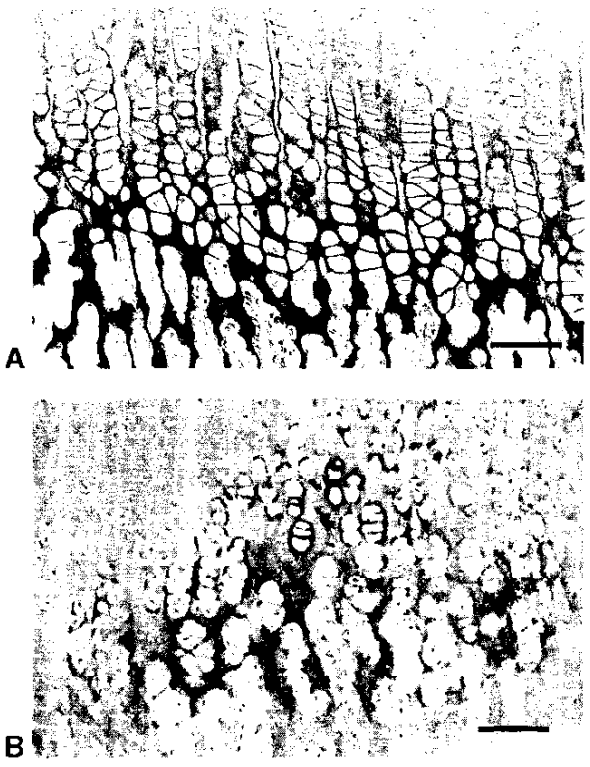

Figure 5. (A) Growth-plate/metaphyseal junction from a control calf stained to show the distribution of versican. There is a slight increase in the matrix staining intensity around hypertrophic chondrocytes. Bar = 80 μm. (B) Growth-plate/metaphyseal junction from a vitamin A-treated calf demonstrates focally increased interterritorial matrix staining around some columnar cartilage cells. Bar = 80 μm.

teoid were heavily stained, but the bone itself was unstained. There was heavy staining of marrow elements.

Type II collagen. There was generalized staining of growth-plate cartilage with heavy focal staining of chondrocyte capsule and territorial matrix within the reserve and columnar cartilage zones. Staining of interterritorial matrix was less strong. There was intense staining of cartilage within osseous trabeculae, but the bone was unstained (**Figure 8**A).

Osteopontin. The cytoplasm of hypertrophic chondrocytes and the surrounding matrix was stained as was cartilage within the trabeculae of the primary spongiosa. At the growth-plate/metaphyseal junction in the zone of cartilage removal, there was heavy staining of the reversal line that formed on trabecular surfaces of the primary spongiosa. Osteoblasts were heavily stained, but bone was only lightly stained (**Figure 9**A). In mature trabeculae, cement lines were prominently stained as were osteocyte canaliculi. Eroded trabecular surfaces were more deeply stained than inactive trabecular surfaces. Formation surfaces showed staining of osteoid surfaces in a fibrillar pattern.

Osteocalcin. The growth-plate matrix was unstained, but there was heavy staining of hypertrophic cartilage cell cytoplasm in both the epiphyseal and metaphyseal growth regions. Osteoblasts were heavily stained, and osteoid and bone were moderately stained (**Figure 10**A). Bone reversal lines, eroded bone surfaces, and osteocyte canaliculi were distinctly delineated. Marrow components were strongly stained.

Osteonectin. Intense cytoplasmic staining of hypertrophic chondrocytes of epiphyseal and metaphyseal growth zones was found. Also, there was heavy staining of territorial matrix of hypertrophic chondrocytes (**Figure 11**A). The chondro-osseous interface of trabeculae within the primary spongiosa was heavily stained. Osteoblasts and other marrow cells were slightly stained. Bone of trabeculae was slightly stained, and there was distinct staining of bone canaliculi, eroded surfaces, and reversal lines.

Bone sialoprotein. Cytoplasm of the cartilage cells within the reserve zone were stained. There was slight staining of hypertrophic cartilage cell cytoplasm and focal staining of chondrocyte capsules (**Figure 12**A). A portion of the territorial matrix along the longitudinal alignment of columnar cartilage cells was heavily stained. Osteoblasts and osteoclasts were heavily stained, and osteoid was lightly stained. There was heavy staining along the osteoid-cartilage interface along the primary spongiosa. The bone itself was poorly stained, but reversal lines, osteocyte canaliculi, and eroded trabecular surfaces were heavily stained.

Alkaline phosphatase. Most of the chondrocytes in the growth plate did not stain. There was heavy cytoplasmic staining of hypertrophic chondrocytes at the epiphyseal and metaphyseal chondro-osseous interfaces. There was intense staining of the capsule of hypertrophic chondrocytes at the metaphyseal border

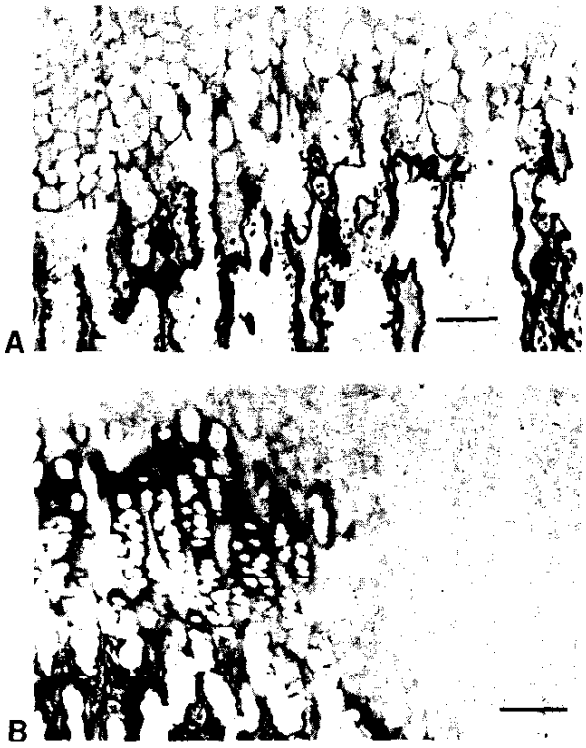

Figure 6. (A) Growth-plate/metaphyseal junction from a control calf stained to show distribution of type I collagen. Reaction product can be seen localized to the bone on the surface of cartilage trabeculae of the primary spongiosa. Osteoblasts are lightly stained. Bar = 80 μm. (B) Growth-plate/metaphyseal junction from a vitamin A-treated calf stained to show distribution of type I collagen. In the region where the growth plate has become focally thin, reaction product is seen focally in the matrix surrounding chondrocytes within the columnar cartilage. Bar = 80 μm.

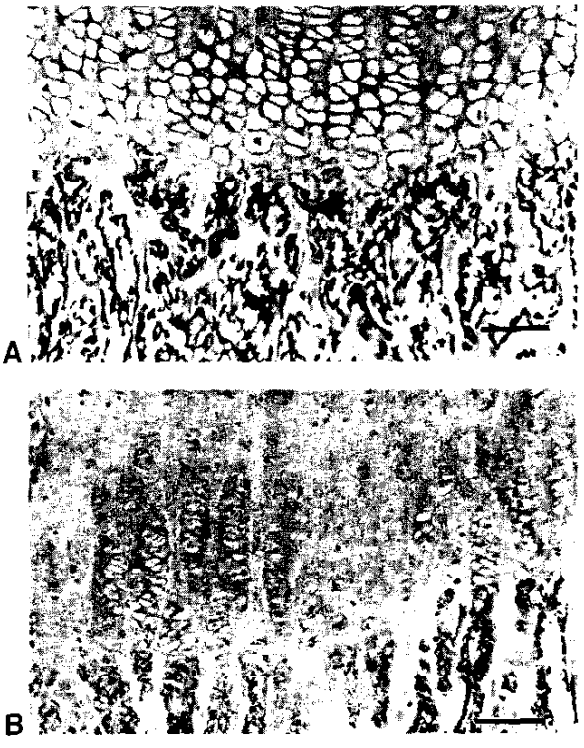

Figure 7. (A) Growth-plate/metaphyseal junction from a control calf stained to show distribution of type I collagen propeptide. Cytoplasmic staining and increased capsular staining is present in the hypertrophic cartilage zone. Osteoblasts are heavily stained, and osteoid, but not bone, is moderately stained. Bar = 80 μm. (B) Growth-plate/metaphyseal junction from a vitamin A-treated calf demonstrates the distribution of type I collagen propeptide. The columnar cartilage cells have spotty accumulation of cytoplasmic staining, and osteoblasts are heavily stained. Bar = 80 μm.

and within the territorial matrix that ran along a longitudinal direction (**Figure 13**A). Perichondral granular deposits were seen. Bone was unstained, but osteoblasts were heavily stained. A double line of positive staining was seen where eroded bone surfaces were adjacent to an osteoclast border (Figure 13B).

Week 1: Treated

Histology showed that the growth plate varied in depth along its width. Some regions were relatively thick, but there were local areas where the thickness was moderately reduced or where the plate was very thin. The depths of various cartilage zones within these regions are shown in Table 1. As the growth plate became thinner, the thickness of both the reserve and columnar cartilage zones was reduced. When the growth plate was very thin, the ratio of the thickness of the columnar cartilage zone to the total reserve zone was about equal to that found in the control animal. As the thickness of the columnar cartilage zone became reduced, the cartilage cells maintained an appearance similar to maturing chondrocytes of the control growth plate. Enlarged cells with typical hypertrophic chondrocyte morphology usually were not seen. Mineralization of the columnar cartilage occurred along the interterritorial matrix that ran in a longitudinal direction adjacent to the nonhypertrophic cartilage cells of the columnar cartilage. In focal areas, the mineralization extended for a greater depth than normal along the cartilage cell column (Figure 1B). The mineralized cartilage spicules of the primary spongiosa were shorter and thinner than those of the control animal. As noted previously,[44] there was loss of metachromatic staining within a narrow subepiphyseal band that constituted the epiphyseal growth zone and an adjacent portion of the reserve cartilage zone.

Immunohistochemistry. Staining for aggrecan demonstrated focal loss within reserve cartilage matrix along the epiphyseal border (Figure 2B). There was some retention of capsular aggrecan staining around chondrocytes. Loss of aggrecan staining occurred in the same region where loss of metachromasia was observed with toluidine-blue staining. Focal loss of another smaller proteoglycan, decorin, from this region was also apparent (Figure 3B). In zones where the growth plate was thin, there was reduction in the intensity of staining for decorin within the interterritorial matrix of the columnar cartilage zone. The staining for a third proteoglycan, biglycan, in the reserve cartilage was light, however, the intensity of staining of the interterritorial matrix of the columnar cartilage zone was increased and similar to that observed in control animals (Figure 4B). There was focally increased territorial matrix staining of the second large proteoglycan, versican, in the columnar cartilage cell zone (Figure 5B). Type I collagen antibody that recognizes the helical domain of the processed collagen trimers demonstrated increased staining of the territorial matrix within columnar cartilage that

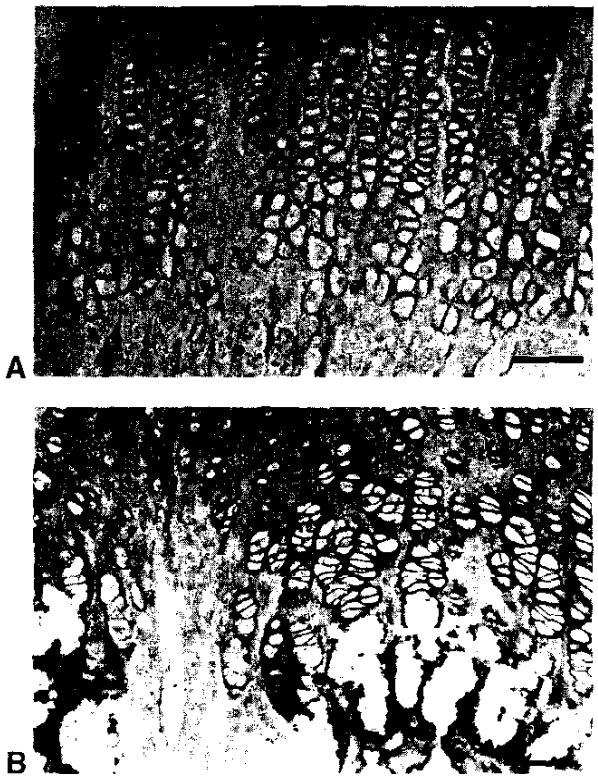

Figure 8. (A) Growth-plate/metaphyseal junction from a control calf stained to show the distribution of type II collagen. The chondrocyte capsule within the zone hypertrophic chondrocytes is stained. Bar = 80 μm. (B) Growth-plate/metaphyseal junction from a vitamin A-treated calf demonstrates localization of type II collagen similar to that seen in control calf (Figure 8A). There is slight increased staining intensity of cartilage cores within the zone of cartilage removal. Bar = 80 μm.

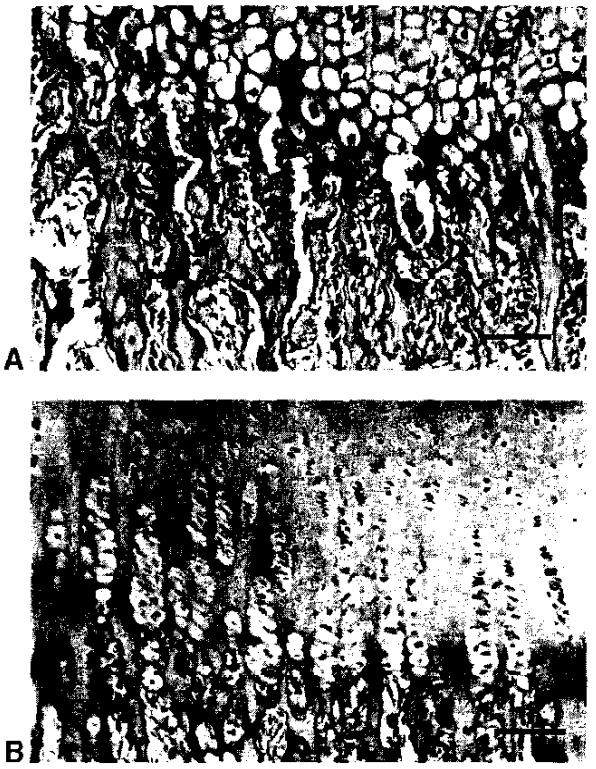

Figure 9. (A) Growth-plate/metaphyseal junction from a control calf demonstrates localization of osteopontin. Staining is seen within the cytoplasm and matrix surrounding hypertrophic chondrocytes. Note staining of osteoblasts and heavy staining of reversal line at the cartilage bone interface within the primary spongiosa. Bar = 80 μm. (B) Growth-plate/metaphyseal junction from a vitamin A-treated calf demonstrates localization of osteopontin. There is a focus where staining intensity is increased in a longitudinal region of pericellular matrix adjacent to undifferentiated columnar cartilage chondrocytes. Bar = 80 μm.

extended deeper into the columnar zone and sometimes surrounded the columnar cartilage arcades (Figure 6B). We also used an antiserum that binds to the propeptide of type I collagen to illustrate the cells synthesizing the collagen matrix. The columnar cartilage cells had spotty accumulation of cytoplasmic staining of the propeptide (Figure 7B). The distribution of stain for type II collagen in the columnar cartilage zone was similar to that of control animals except the intensity of staining of cartilage remaining in trabeculae in the zone of cartilage removal was more intense (Figure 8B). At the growth-plate/metaphyseal border in the zone of cartilage removal, reaction product with osteopontin heavily stained the reversal line between cartilage and the osteoid deposited on trabecular surfaces of the primary spongiosa. The interterritorial matrix of the maturing cartilage cells was more deeply stained at the cartilage-metaphyseal interface, but there were multifocal patches where less differentiated columnar chondrocytes had heavy staining of the pericellular matrix at the lateral apical borders (Figure 9B). Osteocalcin was seen within cytoplasm of reserve zone cells, and there was increased variable staining within rows of columnar chondrocytes (Figure 10B). Osteonectin was heavily deposited in the pericellular matrix at the lateral apices of maturing chondrocytes and appeared as accumulations of matrix granules throughout the columnar cartilage zone (Figure 11B). Deposition of osteonectin within the cartilage spicules of the primary spongiosa also appeared granular. Antibodies to bone sialoprotein were localized within chondrocyte cytoplasm of nonhypertrophic chondrocytes of the columnar zone (Figure 12B). Reaction product showed increased deposition in chondrocyte capsules, and appeared in a granular fashion in the interterritorial matrix. Alkaline phosphatase had a granular deposition at the poles of nuclei throughout the columnar cartilage zone. Deposition of alkaline phosphatase within the interterritorial matrix was present as a granular pattern (Figure 13B).

Week 2: Control

No substantial differences were noted between the 1 and 2 week control animals.

Week 2: Treated

The growth plate along a broad region had multiple foci where the growth plate was extremely thin or almost absent. As noted previously in the 1 week animal, there was a lack of metachromasia in a linear portion of the reserve zone that ran parallel and adjacent to the epiphyseal growth zone. The matrix in this region had increased basophilia, and there appeared to be increased clustering of chondrocytes. The columnar cartilage on the metaphyseal side of the reserve zone was extremely thin. It some-

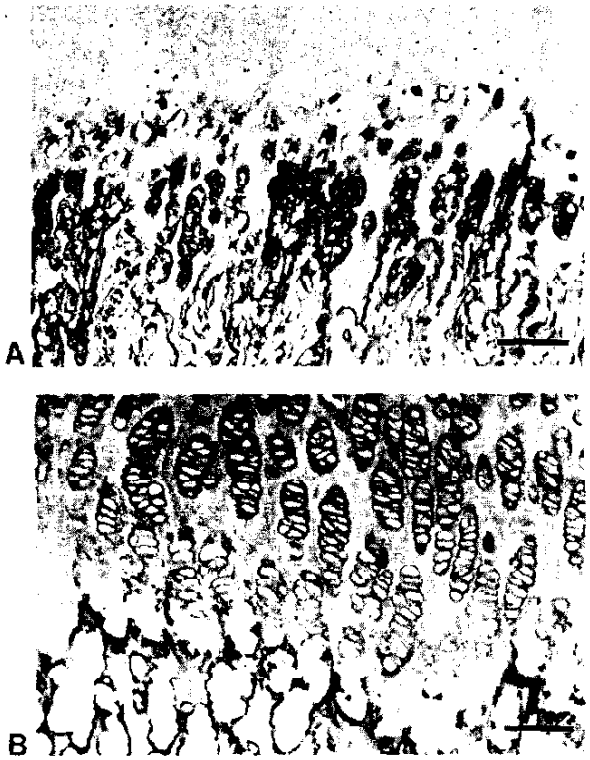

Figure 10. (A) Growth plate/metaphyseal junction from a control calf demonstrates localization of osteocalcin. The growth plate matrix is unstained, but there is staining of hypertrophic cartilage cell cytoplasm. Bar = 80 μm. (B) Growth-plate/metaphyseal junction from a vitamin A-treated calf. Bone within the primary spongiosa is stained, and there is increased moderate staining of chondrocyte capsules within columnar chondrocytes. Bar = 80 μm.

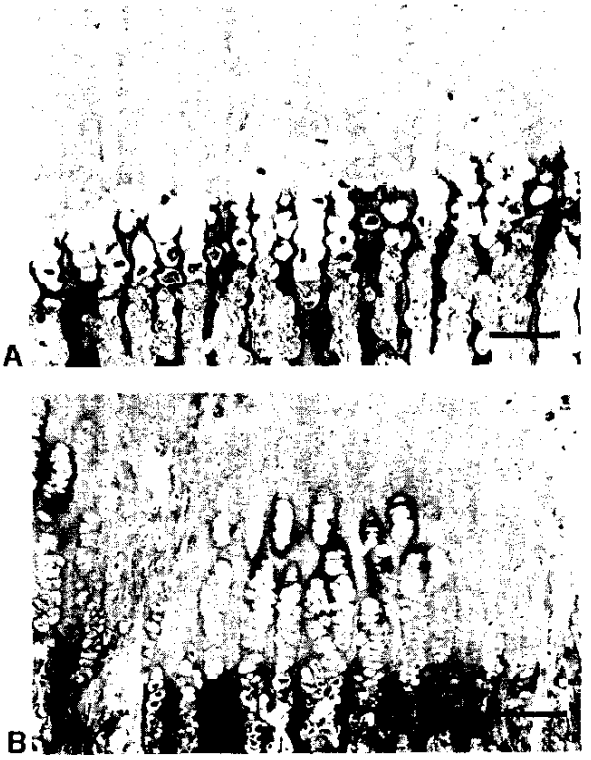

Figure 11. (A) Growth-plate/metaphyseal junction from a control calf stained to show distribution of osteonectin. Heavy deposits of reaction product are localized along the surface of cartilage trabeculae of the primary spongiosa. Reaction product is also evident in the cytoplasm of hypertrophic chondrocytes and in the territorial matrix lining the longitudinal septae. The interterritorial matrix of the longitudinal septae stained slightly. Bar = 80 μm. (B) Growth-plate/metaphyseal junction from a vitamin A-treated calf stained to show distribution of osteonectin. A focally thin region of cartilage shows reaction product with normal distribution along cartilage trabecular surfaces of the primary spongiosa. Heavy deposits are localized in the territorial matrix of the nonhypertrophic chondrocytes of columnar cartilage and can be seen in the reserve cartilage zone. Bar = 80 μm.

times consisted of only a few cells in longitudinal parallel rows, or it was completely absent. In some regions, there was almost complete conversion of reserve cartilage into columnar cartilage. Mineralization of growth-plate cartilage extended along the interterritorial matrix in a longitudinal direction between rows of chondrocytes of the columnar zone. There were also regions where the reserve cartilage zone was undergoing mineralization. The spicules of mineralized cartilage that now formed the primary spongiosa appeared rudimentary with multiple interconnections.

Immunohistochemistry. There was decreased staining for three of the proteoglycans (aggrecan, decorin, and biglycan) in cartilage where loss of metachromasia was demonstrated. In general, the localizations of matrix proteins were similar to that described in the 1-week calf, and only differences are noted. Type I collagen immunohistochemistry showed increased capsular staining and interterritorial matrix staining in the region of the reserve cartilage where aggrecan was diminished. There also was increased focal capsular and interterritorial matrix staining of columnar cartilage. Chondrocyte cytoplasm throughout the growth plate was stained by antibody to type I collagen propeptide. There was increased capsular and interterritorial matrix staining in the region of aggrecan depletion. Other regions of the reserve zone only had cytoplasmic staining. The interterritorial matrix of the columnar zone was also stained for type I collagen propeptide. Where the growth plate was very thin, there was more diffuse staining for osteopontin within the pericellular matrix of chondrocytes within the reserve zone. Along the epiphyseal chondro-osseous junction, clusters of chondrocytes had very dense staining of the territorial matrix for osteocalcin, and there were very dense granular deposits in the interterritorial matrix of the reserve zone. There was large focal, granular deposition of osteonectin in chondrocyte capsules and interstitial matrix within the reserve zone. Bone sialoprotein stained the capsule and territorial matrix of cartilage adjacent to the epiphyseal border. In addition, regions of the reserve cartilage had granular deposits in the capsule or interterritorial matrix surrounding most of the cells. Also, antibodies to alkaline phosphatase were localized in the cartilage cells of the columnar zone with heavy staining of the interterritorial matrix.

Week 3: Control

Histologic and immunohistochemical findings were similar to those previously described.

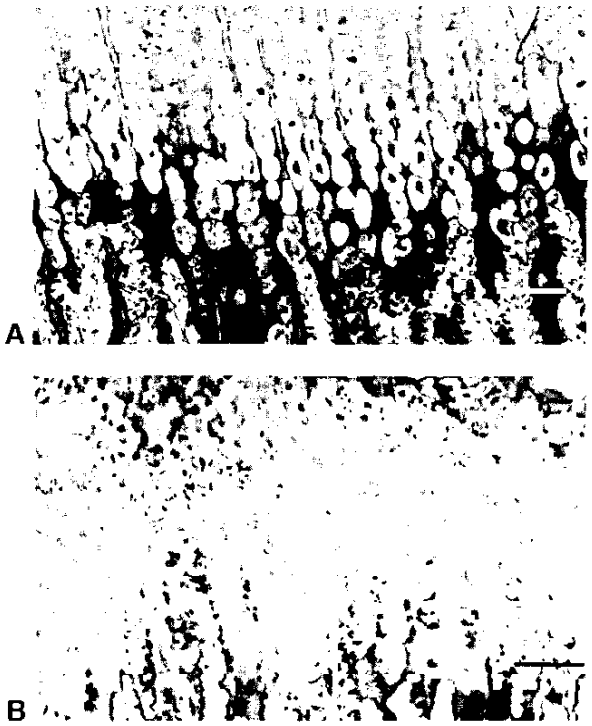

Figure 12. (A) Growth-plate/metaphyseal junction from a control calf stained to show distribution of bone sialoprotein. Reaction product can be seen localized to cartilage trabeculae with heavy deposits on the surface of the primary spongiosa. In addition, there is localization within cartilage cells and matrix of longitudinal septae adjacent to hypertrophic chondrocytes. Bar = 80 μm. (B) Growth plate/metaphyseal junction from a vitamin A-treated calf stained to show distribution of bone sialoprotein. There are granular deposits in chondrocytes and surrounding matrix throughout the growth plate. Bar = 80 μm.

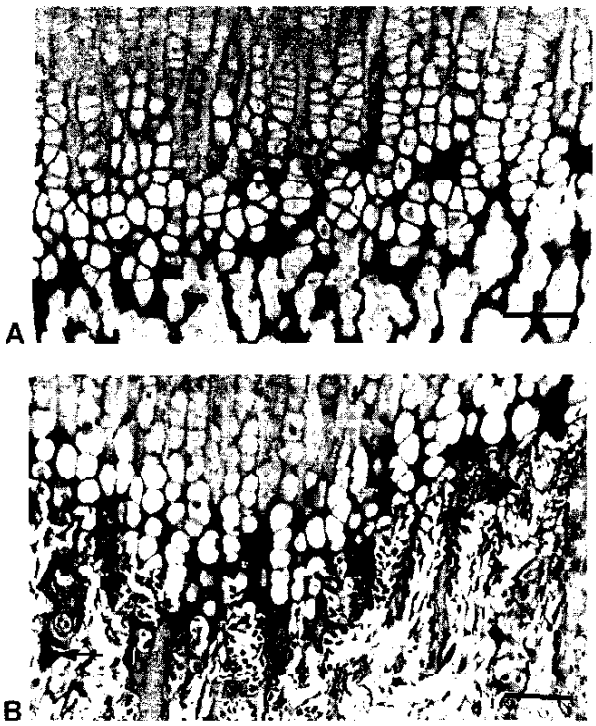

Figure 13. (A) Growth-plate/metaphyseal junction from a control calf stained to show distribution of alkaline phosphatase. Matrix staining is present in the zone of hypertrophic chondrocytes. Bar = 80 μm. (B) Growth-plate/metaphyseal junction from a vitamin A-treated calf. Territorial matrix staining at the chondro-osseous interface is increased. Note normal positive staining of osteoblasts and double line of positive staining at eroded bone surface adjacent to osteoclast (arrow). Bar = 80 μm.

Week 3: Treated

A distinct growth plate had disappeared over a broad region. The area occupied by the former growth plate was easily recognized, and the histology differed only slightly between the two animals. In one animal, there was a wide, thin band of reserve zone cartilage that extended horizontally across the metaphysis. The matrix of this residual cartilage did not stain metachromatically with toluidine blue except within a few foci. There were rather extensive areas that were deeply basophilic. The residual cartilage was bordered on the epiphyseal side by remaining hypertrophic chondrocytes and osseous trabeculae of the epiphysis. On the metaphyseal side, there was spongiosa composed of short spicules of cartilage covered by bone. Within the residual cartilage, there were focal sites of growth-plate cartilage reconstruction where it appeared that osteoclastic dissolution of cartilage matrix was followed by bone formation. A similar morphology was seen in the second animal, except the residual cartilage was separated from the spongiosa by a zone of osteogenesis composed of woven bone trabeculae covered with osteoblasts. The trabeculae were arranged in a parallel fashion and oriented in a longitudinal direction. Residual cartilage was mineralized as demonstrated with von Kossa's stain.

Immunohistochemistry. There was spotty staining of the residual cartilage for aggrecan, decorin, and biglycan. Versican was seen in the interterritorial matrix within this area. There was heavy, patchy localization of type I collagen within the territorial and interterritorial matrix of the residual cartilage. Cytoplasm of the residual cartilage cells stained for type I collagen propeptide, and some staining was seen in the interterritorial matrix. Type II collagen was sparsely distributed within the residual cartilage, but was apparent within the cartilage spicules of the spongiosa. Osteocalcin, osteonectin, bone sialoprotein, and alkaline phosphatase showed prominent, patchy staining within the residual cartilage. It should be noted that, within areas of cartilage undergoing reconstruction, dense surface localization of osteopontin, osteocalcin, osteonectin, bone sialoprotein, and alkaline phosphatase was identified beneath some, but not all osteoclasts. The brush border of the osteoclasts had dense localization of reaction product in some instances.

Discussion

Since the demonstration that intravenous injections of papain caused dissolution of rabbit ear cartilage,[8] enzymatic digestion has been considered a major cause of cartilage degradation. Initially, the release of proteolytic enzyme from lysosomal particles was considered responsible for degradation of protein-polysaccharide complexes within the matrix. Also, liberation of lysosomal hydrolytic enzymes was thought to cause the cellular changes in cartilage cultivated in the presence of vitamin A.[14] Although a previous study[11] of hypervitaminosis A in calves did not show growth-plate closure, the findings in this study were similar to changes reported in a number of different mam-

Bone Vol. 21, No. 2
August 1997:171–182

mals.[7,29,43] The cause of premature growth-plate closure was thought to be associated with vitamin A-induced loss of cartilage matrix.[10,16] Until recently, researchers continued to suggest that degradation of extracellular matrix seen with vitamin A toxicity was due to surface-active and membranolytic effects of retinol on lysosomal membranes, which resulted in release of lysosomal hydrolases.

Recently, studies on the action of vitamin A in cell cultures indicate a new proteinase, commonly referred to as "aggrecanase," which cleaves the Glu373-Ala374 bond of the interglobular domain of aggrecan.[21,26,39] Although this single site of cleavage predominates, the responsible enzyme has not been identified.[18,21] Time course digestion of aggrecan showed that the preferred site of neutrophil collagenase was at the predominant metalloproteinase site before cleavage occurred at the "aggecanase" site. Therefore, the possibility remains that "aggrecanse" may be a metalloproteinase in cartilage.[17]

The in vivo distribution of aggrecan loss from the growth plate in the present study is in contrast to tissue culture studies in which retinoic acid caused aggrecan proteolysis with the release of greater than 90% of the cell-layer aggrecan.[26] Vitamin A-induced loss of aggrecan in calves was limited to a linear region along the epiphyseal side of the reserve cartilage zone and focally around epiphyseal vessels and columnar cells at the chondro-osseous junction. It is likely that this distribution of aggrecan proteolysis resulted because of limited vascularization of the growth plate and mineralization of cartilage at the metaphyseal junction. When retinoic acid-induced catabolism of aggrecan was studied in bovine growth-plate explants, hypertrophic cells were the most responsive and resting cells responded least.[35] It is also possible that so-called "aggrecanase" causes hydrolysis of other cartilage proteoglycans as well. Although growth-plate concentrations of decorin and biglycan are much less than aggrecan, immunostaining for both of these smaller proteoglycans was reduced in the region where aggrecan was lost. It is doubtful that the regional loss of proteoglycan from the growth plate was a major factor in the pathogenesis of premature growth-plate closure, because this region was the vestige of cartilage that remained when growth-plate closure was almost complete.

The predominant alteration that occurred within the growth plate was premature mineralization of columnar chondrocyte and subsequent endochondral ossification. This effect was induced in calves by increasing the oral dose of vitamin A.[44] Thus, the effects of vitamin A in calves were not unexpected because they are similar to the in vitro effects of retinoic acid in which rapid mineralization and expression of mineralization-related genes were induced.[11,22,23] This process was best visualized at 1 week when inhibition of longitudinal bone growth and thinning of the growth plate occurred.[44] Bone elongation on the metaphyseal side of the growth plate is dependent on mitotic division within the proliferative zone and cellular enlargement and interstitial deposition of cartilage matrix. Neither of these processes was sufficient to maintain normal growth-plate depth. This was because columnar chondrocytes failed to attain typical hypertrophic-chondrocyte morphology, terminal columnar cartilage cells were closely packed and lacked significant amounts of intervening territorial matrix, and the number of cells within the columnar-cartilage zone decreased (Figure 1A, B). Retinoic acid is reported to cause proliferation of chondrocytes in murine dorsal longitudinal ligament.[11] This may represent a species difference or the effect of age or anatomical location; hypervitaminosis is known to cause vertebral hyperostosis in adult humans and felines.

Comparison of the depth of various growth-plate zones between thin regions from 1 week treated and normal growth plate from the control animal (Table 1), revealed the following: Initially, reduction in growth-plate depth occurred because of cell loss from the columnar cartilage zone (treated wide vs. control; Table 1). But as the growth plate became thinner (treated, medium and narrow vs. control), there was a decrease in the depth of both the columnar and reserve cell zones. Finally, when the growth plate became very thin, it was apparent that the depth of the columnar cartilage zone was being maintained at the expense of the depth of the reserve cartilage. In the very narrow growth-plate regions (narrow, treated vs. control), the total depth of the growth plate was not as thick as the reserve cell zone from the control. Yet, the narrow region maintained an equal ratio of columnar cartilage depth to reserve cartilage depth. With respect to bovine growth-plate closure in hypervitaminosis A, a portion of the reserve zone lives up to its name and furnishes cells that form columns. It is interesting to note that tissue culture studies have identified thyroxine as a factor that regulates morphogenesis of columnar cartilage,[3] and thyroid hormone and retinoic acid have been shown to induce gene expression through a common response element.[9]

Premature mineralization of columnar cartilage and subsequent endochondral ossification occurred despite the fact that columnar chondrocytes usually did not obtain a hypertrophic morphology. Nevertheless, immunostaining revealed localization of matrix proteins, normally considered markers for hypertrophic chondrocytes, in the cytoplasm and/or territorial matrix of immature-appearing chondrocytes. Because there was positive staining of matrix for biglycan and decorin in the zone of hypertrophic cartilage, the localization of these small glycoproteins in the calf growth plates was slightly different from that previously reported in the human fetus.[5] The expression of alkaline phosphatase, osteocalcin, osteonectin, and bone sialoprotein genes by hypertrophic chondrocytes has been noted previously, and each of these proteins is thought to play a role in cartilage matrix mineralization.[6,30,32,38] It is apparent that a hypertrophic morphology is not requisite for chondrocytes to synthesize matrix proteins characteristic of a mature cell phenotype and to undergo matrix mineralization. After mineralization of the columnar-cartilage interterritorial matrix that surrounded the arcades, mineralization occurred within the reserve zone. Here, again, the chondrocytes in the mineralizing region did not have a typical hypertrophic cell morphology, although some appeared moderately enlarged. There was immunostaining for proteins normally associated with cartilage matrix mineralization. Type I collagen has also been observed in the extracellular matrix of hypertrophic cartilage,[33] but it was suggested that synthesis only occurred in cells arising from vasculature or from cells presumed to be osteoblasts or their precursors.[38] It seems unlikely that the capsular and interterritorial matrix immunolocalization of type I collagen within the reserve zone could have arisen from osteoprogenitor cells or vascular elements.

The discovery that nuclear retinoic acid receptors (RARs) act as inducible enhancer factors provides a basis for understanding how retinoic acid signals could be transduced at the level of gene expression.[37] During the later stages of embryonic development, mRARτ transcripts become specific to the cartilage cell lineage and to differentiating skin.[13] Vitamin A metabolites could initiate growth-plate closure by upregulation of the RARτ gene. This receptor could regulate gene transcription from either a retinoic acid-responsive element or a retinoid X-responsive element within the promoter of specific genes, to cause premature growth-plate mineralization. Although a retinoic acid response element has been identified for osteocalcin,[9] there was no selective expression of this matrix protein in the nonhypertrophic chondrocytes of the columnar zone. Rather, increased staining of nonhypertrophic chondrocytes occurred for most proteins nor-

mally associated with hypertrophic cartilage. These results could indicate that vitamin A-induced expression of chondrocyte proteins occurs by activation of other pathways. Protooncogene expression represents a potential pathway,[27,41] but the osteocalcin gene promoter stimulated by vitamin A was inhibited by the cell proliferation factors jun-fos.[2] A novel pathway, independent of nuclear-receptor-mediated transactivation, has been identified as the possible mechanism for transcriptional regulation during retinoic acid-induced cell differentiation.[24,25]

Transdifferentiation of hypertrophic chondrocytes into bone forming cells has been described in explants of hypertrophic chondrocytes.[36] The transdifferentiated cells within the lacunae had characteristics of osteoblasts, such as alkaline phosphatase activity and positive immunocytochemical staining for osteocalcin, osteonectin, osteopontin, and type I collagen. During growth-plate closure in calves, a similar process was observed in the residual reserve cartilage and adjacent hypertrophic cartilage that did not become columnar cartilage. Here, the reserve cartilage developed specific phenotypic markers that were found in matrix and chondrocytes where calcification and subsequent vascularization occurred. The cartilage cells were not considered to be transformed into osteoblasts, although the residual cartilage lacked metachromasia and had patchy basophilic zones with toluidine-blue staining as well as the aforementioned matrix characteristics for transdifferentiated cartilage. Immunostaining showed that the tissue was not typical of bone. The matrix still contained type II collagen, bone matrix proteins lacked the uniform staining of bone, and cells within the lacunae did not have the canalicular network of osteocytes. This region was eventually removed by a modeling/remodeling mechanism similar to that occurring within the metaphysis.

References

1. Anonymous. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology. New York: McGraw-Hill; 1968.
2. Anonymous. The ostecalcin gene promoter: Stimulated by vitamins A and D, inhibited by cell-growth factors Jun-Fos. Nutr Rev 49:308–309; 1991.
3. Ballock, T. R. and Reddi, A. H. Thyroxine is the serum factor that regulates morphogenesis of columnar cartilage from isolated chondrocytes in chemically defined medium. J Cell Biol 126:1311–1318; 1994.
4. Benya, P. D. and Padilla, S. R. Modulation of the rabbit chondrocyte phenotype by retinoic acid terminates type II collagen synthesis without inducing type I collagen: The modulated phenotype differs from that produced by subculture. Dev Biol 118:296–305; 1986.
5. Bianco, P., Fisher, L. W., Young, M. F., Termine, J. D., and Robey, P. G. Expression and localization of the two small proteoglycans biglycan and decorin in developing human skeletal and non-skeletal tissues. J Histochem Cytochem 38:1549–1563; 1990.
6. Bianco, P., Fisher, L. W., Young, M. F., Termine, J. D., and Robey, P. G. Expression of bone sialoprotein (BSP) in developing human tissues. Calcif Tissue Int 49:421–426; 1991.
7. Cappellin, M. and Crepax, P. Azione della vitamin A in forti dosi sul sistema osseo del ratto albino. Boll Soc Ital Biol Sper 26:1495–1497; 1950.
8. De Duve, C. Subcellular Particles. New York: Ronald; 1959; 128.
9. De Luca, L. M. Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. FASEB J 5:2926–2933; 1991.
10. Dickson, I. and Walls, J. Vitamin A and bone formation. Effect of an excess of retinol on bone collagen synthesis in vitro. Biochem J 226:789–795; 1985.
11. Dietz, U., Aigner, T., Bertling, W. M., and von der Mark, K. Alterations of collagen mRNA expression during retinoic acid induced chondrocyte modulation: Absence of untranslated alpha 1(I) mRNA in hyaline chondrocytes. J Cell Biochem 52:57–68; 1993.
12. Dodge, G. R., Pidoux, I., and Poole, A. R. The degradation of type II collagen in rheumatoid arthritis: An immunoelectron microscopic study. Matrix 11:330–338; 1991.
13. Dolle, P., Ruberte, E., Kastner, P., Petkovich, M., Stoner, C. M., Gudas, L. J., and Chambon, P. Differential expression of genes encoding α, β, γ retinoic acid receptors and CRABP in the developing limbs of the mouse. Nature 342:702–704; 1989.
14. Fell, H. B. and Dingle, J. T. Studies on the mode of action of excess of vitamin A. 6. Lysosomal protease and the degradation of cartilage matrix. Biochem J 87:403–408; 1963.
15. Fisher, L. W., Stubbs, J. T., and Young, M. F. Antisera and cDNA probes to human and certain animal model bone matrix noncollagenous proteins. Acta Orthop Scand 66(Suppl. 266):61–65; 1995.
16. Forsyth, K. S., Watson, R. R., and Gensler, H. L. Osteotoxicity after chronic dietary administration of 13-cis-retinoic acid, retinyl palmitate or selenium in mice exposed to tumor initiation and promotion. Life Sci 45:2149–2156; 1989.
17. Fosang, A. J., Last, K., Neame, P. J., Murphy, G., Knauper, V., Tschesche, H., Hughes, C. E., Caterson, B., and Hardingham, T. E. Neutrophil collagenase (MMP-8) cleaves at the aggrecanase site E373-A374 in the interglobular domain of cartilage aggrecan. Biochem J 304:347–351; 1995.
18. Hardingham, T. E. and Fosang, A. J. The structure of aggrecan and its turnover in cartilage. J Rheumatol 43(Suppl.):86–90; 1995.
19. Hembry, R. M., Knight, C. G., Dingle, J. T., and Barrett, A. J. Evidence that extracellular cathepsin D is not responsible for the resorption of cartilage matrix in culture. Biochim Biophys Acta 714:307–312; 1982.
20. Henderson, C. Aminoalkylsilane: An inexpensive, simple preparation of slide adhesion. J Histotechnol 12:123–124; 1989.
21. Hughes, C. E., Caterson, B., Fosang, A. J., Roughley, P. J., and Mort, J. S. Monoclonal antibodies that specifically recognize neoepitope sequences generated by 'aggrecanase' and matrix metalloproteinase cleavage of aggrecan: Application to catabolism in situ and in vitro. Biochem J 305:799–804; 1995.
22. Iwamoto, M., Shapiro, I. M., Yagami, K., Boskey, A. L., Leboy, P. S., Adams, S. L., and Pacifici, M. Retinoic acid induces rapid mineralization and expression of mineralization-related genes in chondrocytes. Exp Cell Res 207:413–420; 1993.
23. Iwamoto, M., Shapiro, I. M., Yagami, K., Boskey, A. L., Leboy, P. S., Adams, S. L., and Pacifici, M. Retinoic acid induces rapid mineralization and expression of mineralization-related genes in chondrocytes. Exp Cell Res 207:413–420; 1993.
24. Kitabayashi, I., Chiu, R., Umesono, K., Evans, R. M., Gachelin, G., and Yokoyama, K. A novel pathway for retinoic acid-induced differentiation of F9 cells that is distinct from receptor-mediated trans-activation. In Vitro Cell Devel Biol Anim 30A:761–768; 1994.
25. Kitabayashi, I., Eckner, R., Chiu, R., Gachelin, G., Livingston, D. M., and Yokoyama, K. K. Phosphorylation of the adenovirus E1A-associated 300 kDa protein in response to retinoic acid and E1A during the differentiation of F9 cells. EMBO J 14:3496–3509; 1995.
26. Lark, M. W., Gordy, J. T., Weidner, J. R. et al. Cell-mediated catabolism of aggrecan. Evidence that cleavage at the aggrecanase site (Glu373-Ala374) is a primary event in proteolysis of the interglobular domain. J Biol Chem 270: 2550–2556; 1995.
27. Leon, Y., Sanchez, J. A., Miner, C., Ariza-McNaughton, L., Represa, J. J., and Giraldez, F. Developmental regulation of Fos-protein during proliferative growth of the otic vesicle and its relation to differentiation induced by retinoic acid. Devel Biol 167:75–86; 1995.
28. MacKay, R. J., Woodard, J. C., and Donovan, G. A. Focal premature physeal closure (hyena disease) in calves. J Am Vet Med Assoc 201:936–938; 1992.
29. Maddock, C. L., Wolbach, S. B., and Madock, S. Hypervitaminosis A in the dog. J Nutr 39:117–137; 1949.
30. Mandlos, S., Schwahn, B., Reichert, T., and Zabel, B. Distribution of osteonectin mRNA and protein during human embryonic and fetal development. J Histochem Cytochem 40:283–291; 1992.
31. McDonald, J. A., Broekelmann, T. J., Matheke, M. L., Crouch, E., Koo, M., and Kuhn, C. A monoclonal antibody to the carboxyterminal domain of procollagen type I visualizes collagen-synthesizing fibroblasts. Detection of an altered fibroblast phenotype in lungs of patients with pulmonary fibrosis. J Clin Invest 78:1237–1244; 1986.
32. McKee, M. D., Glimcher, M. J., and Nanci, A. High-resolution immunolocalization of osteopontin and osteocalcin in bone and cartilage during endochondral ossification in the chicken tibia. Anat Rec 234:479–492; 1992.
33. Mizoguchi, I., Nakamura, M., Takahashi, I., Kagayama, M., and Mitani, H. An immunohistochemical study of localization of type I and type II collagens in mandibular condylar cartilage compared with tibial growth plate. Histochemistry 93:593–599; 1990.
34. Oettinger, H. F. and Pacifici, M. Type X collagen gene expression is transiently up-regulated by retinoic acid treatment in chick chondrocyte cultures. Exp Cell Res 191:292–298; 1990.
35. Plaas, A. H. and Sandy, J. D. A cartilage explant system for studies on aggrecan

structure, biosynthesis and catabolism in discrete zones of the mammalian growth plate. Matrix 13:135–147; 1995.

36. Roach, H. I. Trans-differentiation of hypertrophic chondrocytes into cells capable of producing a mineralized bone matrix. Bone Miner 19:1–20; 1992.
37. Robertson, M. Retinoic acid receptor. Towards a biochemistry of morphogenesis. Nature 330:420–421; 1987.
38. Sandell, L. J., Sugai, J. V., and Trippel, S. B. Expression of collagens I, II, X, and XI and aggrecan mRNAs by bovine growth plate chondrocytes *in situ*. J Orthop Res 12:1–14; 1994.
39. Sandy, J. D., Neame, P. J., Boynton, R. E., and Flannery, C. R. Isolation and characterization of disulfide-bonded peptides from the three globular domains of aggregating cartilage proteoglycan. J Biol Chem 266:8683–8685; 1990.
40. Sasano, Y., Mizoguchi, I., Kagayama, M., Shum, L., Bringas, P., Jr., and Slavkin, H. C. Distribution of type I collagen, type II collagen and PNA binding glycoconjugates during chondrogenesis of three distinct embryonic cartilages. Anat Embryol (Berl) 186:205–213; 1992.
41. Sharpe, G. R., Fisher, C., and Redfern, C. P. Changes in oncogene mRNA expression during human keratinocyte differentiation. Arch Dermatol Res 286:476–480; 1994.
42. Van Sickle, D. C. and Kincaid, S. A. The articular-epiphyseal cartilage complex. In: Proceedings of the 64th CRAWD, Chicago, IL, 1983; 9.
43. Wolbach, S. B. Vitamin A deficiency and excess in relation to bone growth. J Bone Jt Surg 29:171–192; 1947.
44. Woodard, J. C. J Comp Pathol. To be published.

Date Received: November 8, 1996
Date Revised: March 11, 1997
Date Accepted: April 4, 1997